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Distribution of Cadmium-Binding Components in Flax (*Linum usitatissimum* L.) Seed

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The distribution of cadmium- (Cd-) binding components in flaxseed (cultivar NorMan) containing 0.526 ppm (ng/mg) Cd was investigated. Proteins extracted from dehulled, defatted flaxseed were fractionated by anion-exchange and size-exclusion chromatography. The contents of Cd and other metals, UV/visible spectral characteristics, and amino acid compositions of these fractions were analyzed. Over 66% of the eluted Cd was recovered by 0.1 M NaCl elution from DEAE-Sephacel, in a thiol-rich fraction representing only 7% of the extracted proteins. Sephadex G50 size-exclusion chromatography of this 0.1 M NaCl fraction concentrated most of the Cd in a low-molecular-weight peak eluting at V_t. About 72% of the extracted flaxseed proteins eluted from DEAE-Sephacel at 0.25 M NaCl and contained only 25% of the eluted Cd. Because the major Cd-binding fraction is a minor constituent of flaxseed, these results indicate the potential to isolate flaxseed's major storage protein with a low Cd content.

KEYWORDS: Flax (*Linum usitatissimum* L.); flaxseed; linseed; cadmium; proteins; peptides

INTRODUCTION

Flax (*Linum usitatissimum* L.) is an economically important oilseed crop. Apart from its traditional usage as a raw material in oil production, flaxseed (also called linseed) has turned out to be a functional food ingredient with increasing importance in the world market because of its potential to reduce the risk of cardiovascular diseases, cancers, and gastrointestinal disorders (*1*−*3*).

However, flaxseed often accumulates significant amounts of cadmium (Cd) that exceed the dietary critical value or maximum level of 0.3 ppm (*4*−*7*). The Cd content of 109 accessions of flaxseed from the world collection has been reported to range from 0.075 to 2.775 ppm, with over 40% of the population being higher than 1.4 ppm (*8*). A range of 0.14−1.37 ppm Cd has been reported for flax grown in North America (*9*).

The FAO/WHO provisional guideline level of 0.1 ppm for Cd in flaxseed is currently under debate (*10*). Cadmium is generally considered as a toxic element that can accumulate in the human kidney through the food chain, causing renal tubular dysfunction and pulmonary emphysema. In nonoccupationally exposed populations, the major source of exposure to and intake of Cd are diet and smoking (*11*). Because the half-life of Cd in the human body is about 20 years, the consumption of foods containing higher levels of Cd can result in chronic toxicity (*12*).

For most crops, Cd accumulation in generative organs is minor compared to the accumulation in roots and shoots. However, in flax, a high concentration of Cd is known to accumulate in the seeds even at low soil Cd level. This can be of major importance if flaxseed is used as a food in the diet (*4*). Another important phenomenon is that different genotypes of flax accumulate quite different amounts of Cd in the seeds even when grown under the same conditions (*4*, *9*). However, the underlying mechanism for this genotypic difference, as well as the form of Cd existing in flaxseed, is still unknown.

Normal plant growth and development depends on mechanisms that maintain internal concentrations of essential metals, such as Cu, Ni, and Zn, between limits of deficiency and toxicity and of nonessential metals, such as Cd, Pb, and Hg, below their toxicity thresholds (*13*). Two of the primary biological tolerance systems for modulating Cd stress are metallothioneins (MTs) and phytochelatins (PCs).

MTs are a group of low molecular weight and cysteine-rich proteins or peptides that, in their reduced state, provide thiols for metal chelation. MTs are broadly distributed among animals, eukaryotic microorganisms, certain prokaryotes, and plants (*13*). Numerous reports regarding MTs in animals and bacteria have been issued since the first discovery of a Cd-MT from horse kidney in 1957 (*14*). On the other hand, very few reports have been published on MTs isolated from plant materials. One notable example of an MT from plants is the Ec protein from wheat embryos that contains 12 cysteines in the first 59 residues and binds Zn (*15*).

PCs are a group of nontranslational and cysteine-rich peptides, consisting of repeating units (2−11) of γ-glutamate−cysteine

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with a glycine, β-alanine, serine, glutamic acid, or glutamine at the carboxy terminus (*13*, *16*, *17*). PCs appear to be ubiquitous in the plant kingdom (*18*) and are believed to function as detoxifying agents for Cd by virtue of their ability to bind Cd, thereby preventing it from reacting with the sulfhydryl groups of vital enzymes and proteins.

Because of the minor quantity of Cd typically found in seeds, no direct study on Cd-binding protein(s) in seeds has been reported until recently. Ec protein, Cd-binding, and cysteine-rich proteins in wheat and maize were discovered by investigation of the gene, and other similar metallothionein proteins were discovered by screening of the cDNA library. The route is from gene to protein and then to metal-binding characteristics (*19*).

Normally, the major storage form for minerals in mature seeds is phytin or phytate, a mixed salt of myoinositol hexaphosphoric acid or phytic acid. Phytate can be dispersed throughout the protein matrix or localized into dense aggregates called globoids in the protein body (*13*). Although zinc phytate was identified in roots of crop plants, attempts to isolate and characterize cadmium phytate in roots under conditions of Cd or Cd + Zn exposure have not been successful (*20*), and to our knowledge, the presence of cadmium phytate has also not been confirmed in seeds.

In the case of flaxseed, Cd is not present in the oil and is believed to be bound to proteins (*21*). The protein contents of flaxseed cultivars grown in Canada generally are well above 36% (*22*). Because it was reported that the Cd in rice grains was bound to the major storage protein glutelin (*23*), our laboratory initiated studies to characterize the major storage protein in flaxseed (*24*) and to determine the presence of Cd-binding components in protein extracts of dehulled and defatted flaxseed (*25*). Whereas the major storage protein of flaxseed was isolated in a 0.25 M NaCl fraction by DEAE-Sephacel ion-exchange chromatography (*24*), phytochelatin-like components were found in two fractions eluting at high salt concentrations of 0.45 and 0.50 M NaCl or 35−40 mS/cm (*25*).

Although this previous research determined the presence of Cd-binding components, it focused on the high-salt-eluted fractions, which were present in only trace quantities, and did not provide any insight into the mechanism of the bulk Cd existence in flaxseed. Another limitation of this earlier research was that the ion-exchange column was overloaded with protein to facilitate recovery of the trace amounts of high-salt-eluted fractions. Under these conditions, the real Cd distribution in various protein fractions could not be determined. It has been suggested that the existence of multiple metal-binding proteins having different anionic characters is likely (*25*). Furthermore, Cd can not only be bound to metallothioneins or phytochelatins, but it can also be associated with quite different ligands, such as organic acids, amino acids, and phytin (*13*).

Information regarding Cd-binding components is much needed to understand the mechanism for Cd accumulation in flaxseed. This knowledge might be helpful in future research to develop a marker system for the selection of genotypes with low tendency for Cd accumulation in the seeds. There is also a need to examine a far wider range of flaxseed germplasm to find potential breeding material with lower levels of Cd accumulation in seed. Therefore, intensive research is needed to identify the distribution and mechanism of Cd accumulation in flaxseed to reduce the risk of Cd toxicity and to ensure continued safety with increased flaxseed utilization in food and medicinal industries.

The objectives of our research are to reveal the distribution of Cd in flaxseed proteins and to investigate the feasibility of isolating flaxseed proteins with low Cd content that can then be used as food ingredients. As a long-term objective, this research will also provide useful information on which to base further investigation of the mechanisms of cadmium uptake and retention in flaxseed.

MATERIALS AND METHODS

Reagents. Tris Ultrapure [Tris-(hydroxymethyl)aminomethane] was from ICN Biomedicals, Inc., Costa Mesa, CA. Sodium chloride and hydrochloric acid (ACS certified) were from Fisher Scientific, Nepean, ON, Canada. 2-Mercaptoethanol electrophoresis reagent was from Sigma-Aldrich Canada Ltd., Oakville, ON, Canada. Bicinchoninic acid (BCA) protein assay reagents A and B were from Pierce Chemical Company, Rockford, IL. The deionized distilled water (~18 MΩ) used for all experiments was produced by a Barnstead water purification system. DEAE-Sephacel and Sephadex G50 were purchased from Amersham Pharmacia Biotech, Inc., Baie d'Urfe, PQ, Canada.

Flaxseed. Flaxseed of the cultivar NorMan containing 0.526 ppm Cd was obtained from the Agriculture and Agri-Food Canada Cereal Research Center (Morden, MB, Canada). To facilitate protein extraction and chromatographic separation, flaxseeds were first dehulled as described by Li-Chan et al. (*25*) by using a Strong Scott Barley Pearler fitted with a 2-mm screen (6.25 holes/cm^2) and an air aspirator (*26*) to remove most of the mucilage from the hulls and to reduce the seed stickiness. The dehulled seeds were then ground in a Wiley Mill (Thomas Wiley Mill, Philadelphia, PA) to pass a 1-mm screen. The ground seeds were defatted by two changes of hexane and one change of petroleum ether, 1 h each with magnetic stirring, by using a seed-to-solvent ratio of 1:10 (w/v). The defatted material was then air-dried under a fume hood for at least 4 h.

Protein Extraction. The defatted flaxseed powder (ca. 5 g from 10 g of dehulled seed) was extracted according to the method of Li-Chan et al. (*25*) with 16 volumes of nitrogen-purged extraction buffer (0.1 M Tris + 0.1 M NaCl + 10 mM mercaptoethanol, pH 8.6) at 4 °C for 16 h with constant magnetic stirring. The extract was centrifuged (10 000*g* for 30 min, Sorvall RC5B, Sorvall Instruments, Dupont, CT) at 8−10 °C, and the supernatant was further centrifuged at 27 000*g* for 30 min.

Ion-Exchange (IE) Chromatography. IE chromatography was carried out by using a modification of the procedure of Li-Chan et al. (*25*). The anion-exchange resin DEAE-Sephacel was equilibrated with equilibrating buffer consisting of 0.01 M Tris + 0.01 M NaCl at pH 8.6. The protein extract from 10 g of dehulled seed was diluted 10-fold with distilled and deionized water (dd water) and then mixed and incubated at ambient temperature with the preequilibrated DEAE-Sephacel resin (500 mL) for 1 h with gentle manual stirring. The unbound proteins were washed off with equilibrating buffer. The resin with bound proteins was packed in a column (5.0 × 30 cm). The bound fractions were then eluted by step gradients containing increasing concentrations of NaCl from 0.1 to 1.0 M in 0.01 M Tris buffer at pH 8.6. The flow rate was 5 mL/min, and the fraction size was 20 mL/tube. Fractions were collected and monitored for conductivity and absorbance at 280 nm.

Size-Exclusion Chromatography. The high Cd-binding fraction eluted from IE chromatography with 0.1 M NaCl was freeze-dried and then redissolved in a small volume of dd water. The redissolved protein sample (ca. 80 mg of protein in 15−20 mL) was loaded onto a column (2.5 × 120 cm) of Sephadex G50 gel (500 mL) that was preequilibrated with dd water. The protein was eluted with dd water at a flow rate of 1 mL/min. Fractions of 10 mL/tube were collected and monitored by measuring conductivity and absorbance at 280 nm.

Analysis of Column Fractions. All analyses were performed on the pooled column fractions.

Cd contents in the IE and G50 fractions were measured by ICP-MS with a USN pulse membrane desolvation inlet by Elemental Research Inc. (North Vancouver, BC, Canada). The detection limit for Cd was 0.1 ppb. The metals in the buffer blank were Ca, 3.97 ± 0.91 ppm; Cu, 0.07 ± 0.02 ppm; Zn, <0.03 ppm; and Cd, <0.3 ppb.

The absorbance spectrum was measured by using an ATI Unicam UV/vis spectrometer (Unicam Limited, Cambridge, U.K.). Conductivity

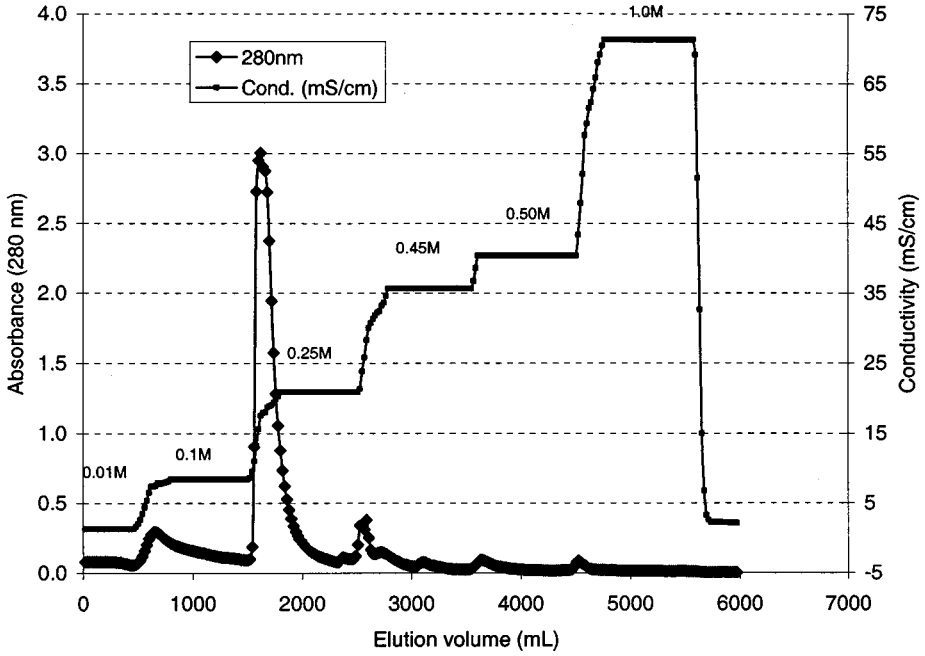

Figure 1. DEAE-Sephacel ion-exchange chromatography of protein extract from dehulled seeds. Proteins were eluted with 0.1–1.0 M NaCl in 0.01 M Tris buffer at pH 8.6.

was measured with a MeterLab CDM 210 conductivity meter (Radiometer Analytical SAS, Lyon, France). Protein content from triplicate samples was determined by using a BCA protein assay kit (#23225, Pierce Chemical Company, Rockford, IL) as well as by a nitrogen combustion method using a LECO FP-428 nitrogen/protein determinator (LECO Instruments Ltd., Mississauga, ON, Canada) calibrated with ethylenediamienetetracetic acid (9.58% × N).

Amino acid analysis was performed by the Advanced Protein Technology Center of Hospital for Sick Children (Toronto, ON, Canada) using a Waters Alliance 2690 separation module. The three fractions of high-molecular-weight (HMW) peak, peak A, and peak B were collected after G50 column chromatography; peak B was dialyzed against deionized and distilled water with a dialysis membrane with a molecular weight cutoff of 100 Da. All three samples were hydrolyzed manually, derivatized with PITC, and then passed through a Pico-Tag RP-HPLC column (3.9 mm × 15 cm) for detection of PTC residues at 254 nm. Cysteine and cystine contents were analyzed as cysteic acid after performic acid derivatization. The data are reported as percentages of residues.

Total SH and total SH + SS contents were determined by using Ellman's reagent [5,5-dithiobis(2-nitrobenzoic acid), DTNB] (*27*) and 2-nitro-5-thiosulfobenzoate (NTSB) (*28, 29*), respectively, in duplicate or triplicate with modifications of the methods as described by Chung (*24*).

RESULTS AND DISCUSSION

Protein and Cd Distributions in Fractions from Protein Extraction Procedure. Dehulling prior to extraction, according to the procedure described by Li-Chan et al. (*25*), alleviated the high viscosity generally associated with mucilage. Extraction of the defatted powder from 10 g of dehulled seed containing 5260 ng of Cd and 2390 mg of proteins with 0.1 M Tris + 0.1 M NaCl at pH 8.6 yielded Cd and protein recoveries of 82 and 56%, respectively (**Table 1**). The Cd and protein recoveries were 86 and 58%, respectively, based on defatted powder, and were deemed appropriate as the achievement of protein recoveries greater than 50% from high-globulin-containing defatted powder has been reported to be very difficult (*30*, *31*).

Protein and Cd Distributions in Fractions from Ion-Exchange Chromatography. The flaxseed protein extract was fractionated by ion-exchange chromatography on a DEAE-Sephacel column (**Figure 1**). The major peak, which eluted at 0.25 M NaCl, was a sharp peak with the highest absorbance value. Two smaller peaks were also observed: a broad peak eluted at 0.1 M NaCl, and a narrow peak containing two shoulders eluted at 0.45 M NaCl. Two minor peaks eluted at 0.5 and 1.0 M NaCl, and sometimes they were not observed in the profiles depending on the quantity of protein loaded onto the column. About 80% of the extracted proteins and 73% of the extracted Cd from flaxseed were bound to the IE column.

Table 1. Cadmium and Protein Contents in Dehulled Seeds, Defatted Powder, and Protein Extract[a]

	Cd		protein		Cd/protein
sample	ng	%	mg	%	ng/mg
dehulled seeds[b]	5260	100.0	2390	100.0	2.2
defatted powder	5010 ± 430	95.2	2297 ± 150	96.1	2.2
protein extract	4320 ± 980	82.1	1330 ± 280	55.6	3.2

[a] Data are the average values ± standard deviation of three separate experiments starting with 10 g of dehulled flaxseed. [b] Data from Agriculture and Agri-Food Canada Pacific Agri-Food Research Center.

Cd was present in the unbound fraction, as well as in each of the 0.1−0.45 M NaCl eluted fractions, suggesting multiplicity of Cd-binding factors (**Table 2**). However, the 0.1 M NaCl eluted fraction was the major Cd-binding peak, containing 66% of the eluted Cd in only 7% of the eluted proteins (or 43% of the Cd in only 3.6% protein, on the basis of dehulled flaxseed). The 0.25 M NaCl fraction was the major protein peak, containing 72% of the eluted protein but only 25% of the eluted Cd (**Table 2**). Results from this experiment indicate the potential to isolate a major storage protein from flaxseed with low Cd content. In contrast, Suzuki et al. (*23*) reported that Cd in rice grain grown in Cd-contaminated fields is present in a form bound to the major storage protein glutelin.

Protein and Cd Distributions in Fractions from Size-Exclusion Chromatography. The major Cd-binding fraction eluting at 0.1 M NaCl from DEAE-Sephacel was further separated into three peaks (**Figure 2**) by size-exclusion chro-

Table 2. Cadmium and Protein Contents in Each Fraction from Ion-Exchange Chromatography[a]

	Cd		protein		Cd/protein
sample	ng	%[b]	mg	%	ng/mg
unbound	285 ± 53	8.3	239 ± 118	18.5	1.2
0.1 M NaCl	2260 ± 830	65.6	86 ± 30	6.7	26
0.25 M NaCl	876 ± 841	25.4	932 ± 154	72.4	0.9
0.45 M NaCl	25 ± 44	0.7	31 ± 31	2.4	0.8
column total	3450	100.0	1290	100.0	

[a] Data are from the average values ± standard deviation of three separate experiments loaded with protein extract from 10 g of dehulled flaxseed. [b] Percentage calculations were based on the total amount eluted from the column.

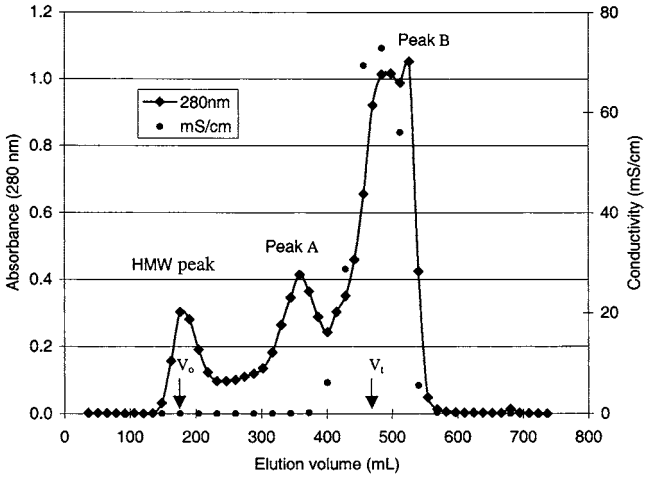

Figure 2. Sephadex G50 size-exclusion chromatography of the IE 0.1 M NaCl fraction eluted in deionized and distilled water.

Table 3. Cadmium and Protein Contents in Each Fraction from G50 Size-Exclusion Chromatography[a]

	Cd		protein		Cd/protein
sample	ng	%[b]	mg	%	ng/mg
HMW peak	ud[c]	N/A[d]	17.3	25.8	N/A
peak A	473	21.3	40.2	60.1	11.8
peak B	1750	78.7	9.50	14.1	184
column total	2220	100.0	67.0	100.0	

[a] Data are from the average values of two separate experiments loaded with the major Cd-binding fraction of 0.1 M NaCl from ion-exchange chromatography starting with 10 g of dehulled flaxseed. [b] Percentage calculations were based on the total amount eluted from the column. [c] ud means under detection limit. [d] N/A means not applicable.

matography on a Sephadex G50 column (fractionation range 1500−30 000). The first peak eluting just after V_o (void volume) was termed the high-molecular-weight peak (HMW peak). The second peak (peak A) eluted at 390 mL, corresponding to a molecular weight of ~14 kDa. The third peak (peak B), eluting after V_t, probably represents low-molecular-weight components including peptides. Both peak A and peak B contained Cd, with peak B being the major Cd-binding fraction, having a high Cd/protein ratio of 184 ng/mg compared to that in dehulled seeds of 2.2 ng/mg (**Tables 1** and **3**). Peak A, with an apparent molecular weight of 14 kDa, is probably an aggregate of low-molecular-weight complexes, because size-exclusion chromatography was performed at low ionic strength where, according to Grill et al. (*32*), metal-containing phytochelatin complexes of 2−4 kDa can aggregate and increase in molecular weight to 10 kDa.

Table 4. Contents of Ca, Cu, and Zn in Each Fraction from Ion-Exchange Chromatography[a]

	Ca		Cu		Zn	
sample	μg	%[b]	μg	%	μg	%
unbound	1010 ± 160	50.7	23.3 ± 20.6	12.8	23.5 ± 9.3	19.6
0.1 M NaCl	217 ± 317	10.9	75.1 ± 60.2	41.2	53.0 ± 22.2	44.1
0.25 M NaCl	333 ± 174	16.7	76.4 ± 20.4	41.9	43.0 ± 5.9	35.8
0.45 M NaCl	433 ± 279	21.7	7.65 ± 2.18	4.2	0.558 ± 0.966	0.5
column total	1990	100.0	182	100.1	120	100.0

[a] Data are from the average values ± standard deviation of three separate experiments loaded with protein extract from 10 g of dehulled flaxseed. [b] Percentage calculations were based on the total amount eluted from the column.

Table 5. Contents of Ca, Cu, and Zn in Each Fraction from G50 Size-Exclusion Chromatography[a]

	Ca		Cu		Zn	
sample	μg	%[b]	μg	%	μg	%
HMW peak	64.3	15.6	20.3	4.8	1.69	2.0
peak A	70.0	16.9	276	65.2	26.5	30.5
peak B	279	67.5	127	30.0	58.7	67.6
column total	413	100.0	423	100.0	86.9	100.1

[a] Data are from the average values of two separate experiments loaded with the major Cd-binding fraction of 0.1 M NaCl from ion-exchange chromatography starting with 10 g of dehulled flaxseed. [b] Percentage calculations were based on the total amount eluted from the column.

Distributions of Ca, Cu, and Zn in Ion-Exchange and Size-Exclusion Chromatographic Fractions. Calcium, copper, and zinc were also monitored along with Cd in each fraction from IE and G50 chromatographies because the toxic effects of cadmium in plants have been shown to be modified by essential minerals such as calcium, copper, and zinc (*33*). It has also been hypothesized that elements with similar physical and chemical properties might act antagonistically to each other biologically, competing for the same transport and storage sites in the cell and displacing each other from reactive enzymatic and receptor proteins (*34*).

The results of metal analyses are shown in **Tables 4** and **5**. Considerable variability was observed between repeated runs. Although the same starting material of dehulled flaxseed was used for these experiments, the dehulled flaxseed was separately defatted and extracted for protein for each run of ion-exchange column chromatography. Thus, some of the variability in the chromatographic profiles might be attributed to slight differences in the concentration and composition of the extracts loaded for chromatography (**Table 1**). A second reason for the variability was that the protein extracts were first mixed with the resin in a batch mode; after incubation and washing, the resin with bound protein was packed into the column for elution. This method greatly decreased the time for sample loading, but it might have resulted in the loss of some protein and bound metals during washing and packing.

Despite these limitations, certain trends can be observed. Calcium was distributed in every fraction of ion-exchange chromatography, with the highest (51%) and lowest (11%) contents being found in the unbound and 0.1 M NaCl fractions, respectively (**Table 4**). After G50 gel filtration chromatography of the 0.1 M NaCl fraction, the majority of Ca was found in peak B, which had a Ca concentration 4 times higher than that in the HMW peak and peak A (**Table 5**). The copper contents were approximately equally distributed (~41%) in the IE 0.1 and 0.25 M NaCl fractions (**Table 4**), and peaks A and B both

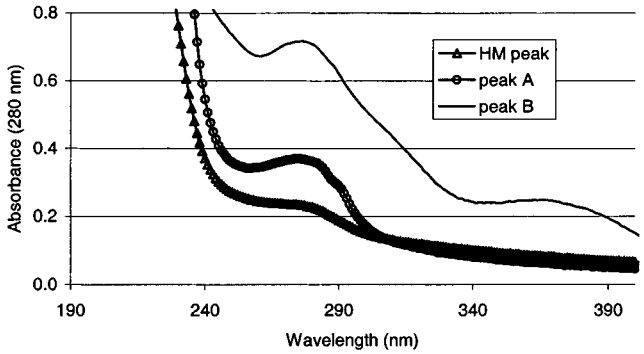

Figure 3. Typical scan profile of each fraction from Sephadex G50 size-exclusion chromatography.

contained large amounts of Cu (65 and 30%, respectively, **Table 5**). According to **Table 4**, most of the Zn was eluted at the medium salt fractions of 0.1 M (44%) and 0.25 M (36%) in ion-exchange chromatography, whereas about 20% of the Zn was eluted with the unbound fraction. In size-exclusion chromatography, peaks A and B had 31 and 68% of the eluted Zn, respectively (**Table 5**).

Cd−Ca, Cd−Cu, and Cd−Zn interactions are commonly observed in plants (*35*). In this research, similar distribution profiles of Zn and Cd were observed in the IE and G50 fractions. The levels of Zn and Cd in decreasing order were 0.1 M, 0.25 M, unbound, and 0.45 M NaCl for the fractions from the IE column, and peak B, peak A, and the HMW peak for the fractions from the G50 column. The highest amounts of both Cd and Zn were thus found in the 0.1 M NaCl fraction and peak B from IE and G50 chromatography, respectively.

In most cases, Zn reduces the uptake of Cd by plants (*35*). Zinc and Cd are chemically similar and might compete for binding sites in the plant (*36*). Studies by Grant and Bailey (*37*) showed a negative relationship between Zn and Cd concentrations in the whole flaxseed. This implies that an active competitive interaction might exist in the seed between Cd and Zn and might suggest a competition for specific carriers. In contrast, in animal studies, a highly positive correlation between Cd and Zn concentrations in liver and kidneys has been noted (*38*). The Cd-induced retention of Zn in the liver and/or kidney is due to Cd accumulation and metallothionein induction in these organs (*39*).

UV/Vis Absorbance Characteristics. UV/visible absorbance spectra of each fraction from ion-exchange and gel-filtration chromatographies were measured, and the wavelength of maximum absorbance (λ_{max}) was noted. The IE 0.25 M NaCl fraction had a peak with λ_{max} of 281 nm, which is typical of proteins. The λ_{max} values for the 0.1 M NaCl and unbound fractions were 276 and 272 nm, respectively, whereas that of the high-salt 0.45 M NaCl fraction was located at a lower wavelength of 267 nm. The 0.1 M NaCl fraction also had a smaller, broad peak between 343 and 373 nm.

For the G50 column fractions, λ_{max} for the HMW peak was near 280 nm. Peaks A and B both had λ_{max} at around 275 nm, but only the major Cd-binding fraction of peak B had an extra peak between 343 and 373 nm, similar to the IE 0.1 M NaCl fraction (**Figure 3**). The unique absorbance spectra of Cd-binding fractions of IE 0.1 M NaCl and peak B might be an indication that these two fractions contained phenolic compounds. Phenolic compounds exhibit two major absorption bands in the UV/visible region: a first band in the range between 320 and 380 nm and a second band in the 250−285 nm range (*40*).

Table 6. SH and SS Contents[a] for Some Fractions from Ion-Exchange and G50 Size-Exclusion Chromatography Experiments

sample	total SH	total SH + SS
protein extract	9.23	82.8
protein extract[b]	N/A[c]	124
0.25 M NaCl[b]	N/A	64.8
0.1 M NaCl	60.5	1104[d]
HMW peak	N/A	15.6
peak A	0.9	38.8
peak B	66.3	272

[a] In micromoles per gram of protein. The values shown are the averages of triplicate measurements. [b] Data from ref *24*. [c] N/A = not analyzed. [d] Average of the data from two separate experiments [1037 and 1170 μmol/(g of protein)], each determined in triplicate.

Table 7. Amino Acid Composition[a] of the Fractions from G50 Size-Exclusion Chromatography

	HMW peak	peak A	peak B
Asp	9.5	5.8	16.3
Glu	14.2	22.4	30.7
Ser	6.1	5.3	5.2
Gly	12.4	16.3	17.0
His	1.6	1.6	0.7
Arg	5.6	7.6	2.3
Thr	5.4	3.7	2.2
Ala	9.8	5.2	1.9
Pro	5.1	2.8	3.9
Tyr	1.9	1.6	2.1
unknown peak 1	–	–	(3.1)[c]
unknown peak 2	–	(8.7)[c]	(82.3)[c]
Val	6.7	4.1	2.4
Met	1.9	1.4	0.5
unknown peak 3	–	–	(7.9)[c]
Cys[b]	1.7	7.1	5.0
Ile	3.6	2.8	3.6
Leu	6.2	5.4	3.1
Phe	2.4	2.0	1.4
Lys	5.9	4.9	1.7

[a] In grams per 100 g of total amino acids. [b] Determined as cysteic acid after performic acid oxidation. [c] Data for the three unknown peaks are the area of each peak expressed as a percentage of the total peak area of all of the amino acids plus the three unknown peaks.

Total SH and Total SH + SS Contents. The total SH and total SH + SS contents are shown in **Table 6**. Because of limited sample sizes, the total SH contents of some fractions were not determined. The estimated contents of SH and SH + SS (in micromoles per gram of protein) for peaks A and B should be considered as approximate values only, because of the low sample concentration and the possible inaccuracy of applying the BCA protein assay for these fractions with unusual compositions. Nevertheless, considering that the average occurrence of cysteine in protein is 1.9% (*41*), corresponding to a calculated total SH + SS content of 157 μmol/(g of protein), the values of 1104 μmol/(g of protein) for the 0.1 M NaCl fraction and 272 μmol/(g of protein) for peak B are very high.

Amino Acid Analysis. The results of amino acid analysis for the three fractions from G50 chromatography, namely, the HMW peak, peak A, and peak B, are shown in **Table 7**. The most abundant amino acids in the three samples were Glu (including Gln) and Gly. In fact, the three amino acids Asp + Glu + Gly comprised 64% of the total amino acids in peak B. The cysteine contents in the three samples were 1.7, 7.1, and 5.0% for the HMW peak, peak A, and peak B, respectively.

The Cd-binding components in different organisms are very complex, and the ligands for Cd are found in various forms,

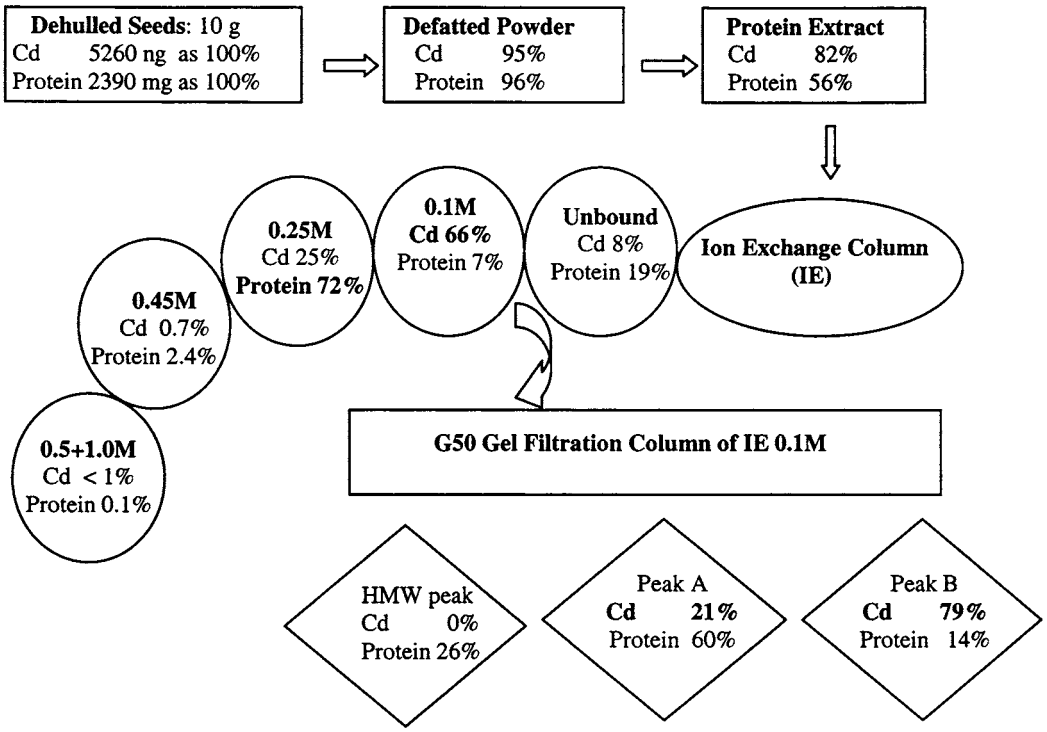

Figure 4. Summary of cadmium and protein recoveries from each step and fraction of isolation and extraction. Percentage recoveries in boxes are expressed on the basis of the total amount in the starting material of dehulled flaxseed; percentages of column fractions are based on the total eluted amount.

including proteins, peptides, amino acids, and other organic acids (*13*). Many of these components show a distant relationship or no relation to the typical horse metallothioneins or phytochelatins, and their cysteine contents vary widely. The cysteine contents in peaks A and B in our study were much higher than the average value of 1.9% for cysteine in proteins (*41*), but still very much lower than the average value of 30% reported for animal metallothioneins (*42*). According to Rauser (*16*), Cys, Glu, and Gly account for 45−97 and 19−92% of the amino acids found in Cd-binding and Cu-binding phytochelatin complexes, respectively, with Cys ranges of 8−43 and 3−39%, respectively. Stone and Overnell (*42*) reviewed a wide range of Cd-binding proteins from various organisms including animals, plants, and microorganisms and concluded that Cd can bind to protein through thiol and nonthiol groups and that the Cys contents in Cd-binding proteins varied from 0 to 34%. A glycoprotein from the mushroom *Agaricus macrosporus* has been characterized and found to contain no Cys, although Glu and Asp together accounted for 35% of the amino acids (*43*). The Cd-binding proteins from whelk were reported to contain aromatic amino acids and only 4−4.9% Cys (*44*).

In addition to the usual amino acids, unknown or unidentified peaks in the chromatographic profiles during amino acid analysis were distinctive features of the peak A and peak B fractions. Both peak A and peak B had an unknown peak at the position of 6.3 min, referred to as unknown peak 2 in **Table 7**, which constituted 8.7 and 82.3% of the total peak area in the amino acid chromatograms of peaks A and B, respectively. Peak B also had two other unknown peaks (unknown peaks 1 and 3 in **Table 7**) at 5.9 and 8.3 min, comprising 3.1 and 7.9%, respectively, of the total peak area. The unknown peaks were not included in the calculation of the percentage of the amino acid residues, but they were, in fact, major constituents, particularly of the peak B fraction. In peak B, considering the high levels of the three unknown peaks (93.3% of total peak area), especially the unknown peak at 6.3 min (82.3%), the composition of amino acids shown in **Table 7** does not represent the actual total composition of peak B. In view of the complexity of Cd-binding components reported to exist in different organisms (*13*, *42*), peak B might contain some very unusual amino acids or other organic acids that can effectively bind Cd. The characterization of these unknown peaks is the subject of ongoing research.

CONCLUSIONS

The techniques of ion-exchange (IE) and size-exclusion chromatography have been used to effectively fractionate the cadmium-binding components from flaxseed (**Figure 4**). The major Cd-binding fraction was eluted at 0.1 M NaCl by IE chromatography, containing 66% of the Cd but only 7% of the total protein eluted from the column. Gel filtration of this IE 0.1 M NaCl fraction separated two Cd-binding fractions with molecular weights of ∼14 000 and <1500, with the latter peak (peak B) as the major Cd-binding fraction. IE 0.1 M and peak B had exceptionally high total SH + SS contents of 1104 and 272 μmol/(g of protein), respectively. However, it is not possible to confirm or eliminate the possibility of phytochelatins and/or metallothioneins as ligands of Cd in the 0.1 M NaCl fraction on the basis of the existing data. Further research is underway to characterize the major components in peaks A and B, to compare the profiles of these components in flaxseed cultivars with varying Cd content, and to evaluate their affinity for binding different metals.

About 72% of the protein loaded onto the IE chromatography column was eluted at 0.25 M NaCl. This IE 0.25 M fraction, corresponding to the major storage protein, contained only 25% of the eluted Cd. These results indicate the potential to isolate the major storage protein in flaxseed with a low Cd content.

ABBREVIATIONS USED

Cd, cadmium; dd, distilled and deionized; G50, Sephadex G50 gel column; IE, ion-exchange column; kDa, kilodalton;

MTs, metallothioneins; MW, molecular weight; PCs, phytochelatins; PITC, phenyl isothiocyanate; ppb, parts per billion; ppm, parts per million; PTC, phenylthiocarbamyl; SH, sulfhydryl; SS, disulfide; RP-HPLC, reverse-phase high-performance liquid chromatography; UV/vis, ultraviolet and visible

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